Neutrophils contribute to fracture healing by synthesizing fibronectin+ extracellular matrix rapidly after injury


Lisa Michels
Journal Club presentation
Introduction: Phases of bone regeneration

→ Fracture hematoma (FH) formation: template for soft callus

→ First cells recruited: polymorphonuclear neutrophils

→ Those attract monocytes/macrophages

# Role of inflammation in bone regeneration

<table>
<thead>
<tr>
<th>positive</th>
<th>negative</th>
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<tr>
<td>Essential for early bone regeneration (inflammatory phase):</td>
<td>Negative effects of certain inflammatory conditions onto fracture healing:</td>
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<tr>
<td>- Removal or irrigation of FH → impaired bone healing</td>
<td>- Inducers of local or systemic inflammation: open fractures, severe soft tissue injury, multiple injuries</td>
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<td>- Transplantation of early FH into muscle tissue → ectopic bone formation</td>
<td>- Experimental models of local/systemic inflammation: LPS-injection intraperitoneal, beta glucan-injection into FH</td>
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Bone histology

- 45% bone minerals, 30% organic matrix, 25% water
- Cells: osteoblasts, osteocytes and osteoclasts
- Mineralized extracellular matrix (ECM):
  - Collagen fibrils (covalent binding mainly of collagen type I fibres)
  - Hydroxyapatite (calcium and phosphate ions)
  - proteoglycans, glycoproteins like fibronectin
  → synthesised by stromal cells like fibroblasts

Fibronectin

• Is an adhesion molecule
• Synthesised by fibroblasts, endothelial cells, macrophages
• Connecting cells and ECM
  • Binding to the cell via integrins
  • Binding to collagen fibrils, fibrin, proteoglykans of the ECM

Hypothesis

• Contribution of inflammatory cells to bone healing by synthesis of an “emergency extracellular matrix (ECM)“

• Before the infiltration of stromal cells, which are synthesizing the actual bone ECM
  (consists mainly of mineralized collagen type I fibrils)
Material and Methods - Outline

• Analysis of temporal changes in the composition of human FH during early bone healing

• → Presence of ECM within FHS isolated before infiltration of stromal cells?

• → Identification of collagen type I + fibrils?

• Isolation of FHS – tissue microarray – analysis of collagen fibers – immunohistochemistry – imaging and analysis
Material and Methods – Isolation of FHs

• 53 FHs of closed fractures of trauma patients were isolated in Open Reduction Internal Fixation procedure

• 4 groups based on the time between injury and isolation

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<td></td>
<td>Within 2 d</td>
<td>3-5 d</td>
<td>6-10 d</td>
<td>&gt;10 d</td>
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<td>15 FHs</td>
<td>10 FHs</td>
<td>15 FHs</td>
<td>13 FHs</td>
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- time ranging from 6h to 23 days
- 18 FHs from upper extremity, 22 FHs from lower extremity, 10 FHs from pelvis, 3 FHs from thorax
- 26 male, 27 female; mean age 45 +/- 19 years

• and 1 control group: coagulated peripheral blood from healthy donors → after 1 h: removed from coagulation tube and treated similar to the freshly isolated FHs
Material and Methods – Isolation of FHs

• Preparation of FHs:

• Directly fixed in 3.7% buffered formaldehyde solution (pH 4) for one week

• Dehydrated and embedded in paraffin (with a Leica Embedding Center)

• Cut into sections of 4μm thickness, incubated at 54°C overnight to allow firm adherence to microscopy slides
Material and Methods – Tissue microarray (TMA)

• Many small representative tissue samples assembled on a single histologic slide → allows simultaneous and comparable staining in one procedure

• Two 1mm cylindrical biopsy cores of each FH were transferred to one TMA paraffin block

• Identification of the biopsy points by staining the sections with hematoxylin and eosin (H&E) with a Dako CoverStainer

• Criteria of the biopsy locations:
  • 1) Area with cells with fibroblast-like morphology
  • 2) Where these cells could not be identified
Material and Methods - TMA

- Sections of 4µm were cut and incubated at 54°C overnight
- Deparaffinisation and rehydration
- Staining:
  - 1) Hematoxylin and eosin
  - 2) Picrosirius Red
  - 3) Immunohistochemistry
Material and Methods – Analysis of collagen fibers – Picrosirius Red

- Collagen fibers are anisotropic and birefringent
- Picrosirius Red enhances their natural birefringence
- TMA sections were deparaffinised, washed with dH2O, stained with PicroSirius Red solution 1h at room temperature (RT)
- Cell nuclei: stained with Hoechst for 10min in the dark at RT
- Dehydration of the sections, embedded with a ClearVue coverslipper and stored in the dark
- Identification of birefringent fibrils: placing the stained sections between two polarization filters with $90^\circ$ of rotational difference between both filters
Materials and Methods - Immunohistochemistry

• 5 TMA sections

• Deparaffinisation, washing with dH2O

• Sequential alkaline phosphatase (ALP) double immunostaining with Liquid Permanent Red Substrate Chromagen and Vector Blue ALP Substrate Kit III

• mouse anti-human primary antibodies, secondary antibodies were BrightVision polyclonal ALP-Anti-Mouse IgG, cell nuclei staining with Hoechst

• Negative isotype control: mouse IgG1
Material and Methods - Immunohistochemistry

- Overview of the five sections and the primary antibodies (ab)

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<th>5-control</th>
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<tbody>
<tr>
<td>1. prim. ab red</td>
<td>leucocytes CD45</td>
<td>neutrophils CD66b</td>
<td>macrophages CD68</td>
<td>neutrophils CD66b</td>
<td>IgG1</td>
</tr>
<tr>
<td>2. prim. ab blue</td>
<td>collagen type I</td>
<td>insoluble cell-derived fibronectin</td>
<td>insoluble cell-derived fibronectin</td>
<td>monocytes and macrophages CD68</td>
<td>IgG1</td>
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- Ab against insoluble cell-derived fibronectin did not recognize soluble plasma-derived fibronectin
Material and Methods - Imaging

• Imaging of each stained TMA core with a Leica DFC425C camera mounted to a Leica microscope
• Imaging of representative images with Olympus DP70 camera connected to Olympus BX51 microscope
• Algorithm to count cell numbers and amount of Vector Blue stained ECM or birefringent fibrils
• Photoshop: merging of blue channel Hoechst images with red channel Liquid Permanent Red images
Results - Group 1: isolation of FH within 2 d

• All nucleated cells were CD45+ leucocytes → majority built of CD66b+ neutrophils and CD68+ monocytes/macrophages
  • No identification of stromal cells

• Significant amount of ECM stained positive for insoluble cell-derived fibronectin
  • Parts of ECM stained mildly positive for collagen type I
  • No identification of birefringent fibrils

• Organization: macrophages mainly localized within fibronectin+ ECM, neutrophils mainly adjacent to the ECM

• Identification of CD66b+ particles within fibronectin+ ECM
Results – Group 2: isolation after 3-5 d

• Day 5 after injury: first identification of stromal cells (CD45-, fibroblast morphology)
  • Macrophages were the most prevalent leukocytes
• ECM majorly positive for fibronectin and increasingly positive for collagen type I
  • No birefringent fibrils
Results – Group 3 & 4: 6-10 d & >10 d

- Majority of nucleated cells were stromal cells
  - Neutrophils decreased further, macrophages remained
- ECM: - strongly positive for collagen type I
  - cellular fibronectin positive in defined areas
  - clear identification of birefringent fibrils
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<th>Group 3 &amp; 4</th>
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<tr>
<td>neutrophils monocytes/macrophages</td>
<td>monocytes/macrophages neutrophils stromal cells (day 5)</td>
<td>stromal cells monocytes/macrophages (neutrophils)</td>
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<tr>
<td>fibronectin (collagen type I)</td>
<td>fibronectin collagen type I</td>
<td>birefringent fibrils collagen type I fibronectin</td>
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Results – Changes in leukocyte counts

Fig. 1. Changes in A) cell count, B) leukocyte count, C) neutrophil counts, D) monocyte count, E) fibronectin/tissue area ratio, F) collagen type 1/tissue area ratio, G) birefringent fibrils/tissue area ratio within human fracture hematomas (FH) over time. The bars represent mean percentage compared to peripheral blood ± standard error of the mean (SEM). The dark gray bars indicate coagulated peripheral blood and the light gray bars show groups of FHs that were isolated at different time-points after injury. Peripheral blood was compared to FHs that were isolated within 48 h after injury. In addition, all FH groups were compared to each other. A p-value < 0.05 is indicated by *, p < 0.01 by **, p < 0.001 by *** and p > 0.05 as ns (not significant).
Results – Changes in ECM composition

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Results – Changes in composition of FH

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<tr>
<td>&lt;3 DAYS</td>
<td>HAEMATOXYLIN &amp; EOSIN</td>
<td>NUCLEUS (Blue) LEUKOCYTE (Red)</td>
<td>FIBRILLAR ECM (PicroSirius Red)</td>
<td>NEUTROPHIL (Red) FIBRONECTIN (Blue)</td>
<td>MACROPHAGE (Red) FIBRONECTIN (Blue)</td>
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Fig. 2. Changes in the composition of the human fracture hematoma over time. Representative images of fracture hematomas (FHs) that were isolated at different time points after injury. Extracellular matrix (ECM) was evident in the early FHs (2A) when practically all nucleated cells were leukocytes (CD45⁺ cells, 2B). Extracellular matrix (ECM) within FHs that were isolated 48 h after injury or later stained positive for cell-derived insoluble fibronectin (2D and 2E). Macrophages (CD68⁺ cells) were mainly localized within the ECM (2E) and neutrophils (CD66b⁺ cells) were mainly localized adjacent to the ECM (2D). During the second week after injury, CD45⁻ cells with a fibroblast-like morphology could be identified within the FH (2 A/2B). After influx of these CD45⁻ cells, birefringent fibrils (2C) became visible within the ECM.
Results - Co-localization of leucocytes and fibronectin

Fig. 2D and 2E: <3 days
Results – Leucocytes and fibronectin

Fig. 3. Localization of innate immune cells in relation to fibronectin+ extracellular matrix (ECM) during the first week after injury. Practically all neutrophils (CD66b+ cells) within coagulated peripheral blood did not stain positive for cellular fibronectin (3A). In contrast, neutrophils stained positive for fibronectin+ in fracture hematomas that were isolated within 48 h after injury and later (3B). Macrophages (CD68+ cells) were predominantly localized within ECM and neutrophils were mainly localized adjacent to the ECM (2C). CD66b+ fragments (2D) could be identified within the ECM during the first week after trauma.
Discussion

• 48h after injury: mainly leucocytes in the FH, fibronectin+ ECM
• Day 5: first appearance of stromal cells, presence of birefringent fibrils

→ Neutrophils as source of fibronectin+ ECM as they stained positive for cellular fibronectin in the FH
  • Neutrophils in coagulated blood stained negative for fibronectin

→ Macrophages might also contribute to fibronectin synthesis
Discussion

- Fibronectin within the fracture gap:
  - EDA+ and EDB+ fibronectin, tenascin-C found in the initial fibrin matrix
  - EDA+ fibronectin and tenascin-C: fracture gap connective tissue
  - Osteofetal EDB+ fibronectin: in osteoblastic cells
- In rheumatoid arthritis patients: higher fibronectin synthesis by polymorphonuclear leucocytes (PMNL) from synovial fluid than by PMNL from peripheral blood

→ Inflammatory stimuli as possible regulators?


Discussion

- CD66b+ particles: possibly secreted microparticles, role in FH remains unclear
  - In vitro: affection of macrophage phenotype: decrease in inflammatory response, increase in TGF-beta1 secretion → local injection of TGF-beta1 improved fracture healing outcome in rats

→ Neutrophils induce regenerative phenotype?

- Systemic depletion of neutrophils: improved outcome
→ Clarify role of neutrophils in (impairment of) fracture healing

- Different neutrophil subsets: CD62L-low and CD11b-high neutrophils both in FH and coagulated blood – coagulation might affect these markers

→ Different subtypes of regenerative and pathogen-battling neutrophils?